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ORIGINAL ARTICLE

Extended-spectrum beta-lactamase-producing bacteria are not detected in supragingival plaque samples from human fecal carriers of ESBL-producing *Enterobacteriaceae*

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Background: The prevalence of infections caused by Cefotaximase-Munich (CTX-M)-type extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-E) has rapidly increased during the past 15 years. *Enterobacteriaceae* are commonly found in the gastrointestinal tract and long-term intestinal carriage is considered important for the spread of ESBL and as a source of clinical infections. Oral biofilm such as supragingival plaque is known to contain numerous antibiotic resistance determinants and may also represent a poorly investigated site for ESBL carriage and further spread.

Objective: To investigate possible carriage of ESBL-producing bacteria in supragingival plaque of known fecal carriers of these bacteria.

Design: We screened for the presence of aerobic and anaerobic ESBL-producing bacteria and $bla_{\text{CTX-M}}$ in supragingival plaque samples from healthy human adults with culture-verified fecal carriage of CTX-M-producing *Escherichia coli*. The presence or absence of *Enterobacteriaceae* and ESBL-producing bacteria in plaque samples was evaluated using culture-based methods and consensus CTX-M PCR.

Results: Oral samples were obtained from 17 participants with known previous carriage of ESBL-producing *E. coli*. No ESBL-producing bacteria or ESBL genes were detected using culture-based and molecular methods. One colony of *Rahnella aquatilis* harboring the class A ESBL gene $bla_{\text{RAHN-1/2}}$ was identified in an oral sample from one of the participants.

Conclusion: This pilot study supports the notion that the presence of CTX-M-producing bacteria is uncommon in oral plaque of healthy human adult fecal carriers. Due to the limited number of persons tested, a low prevalence of oral ESBL-carriage in healthy adults or carriage in selected groups of patients cannot be excluded. To our knowledge, this is the first description of an *R. aquatilis* with the RAHN-1/2 gene in the oral cavity.

Keywords: *CTX-M*; *oral carriage*; *extended-spectrum* β-*lactamase*; *Enterobacteriaceae*

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The spread of genes coding for CTX-M-type extended-spectrum beta-lactamases (ESBLs) among *Enterobacteriaceae* has increased dramatically worldwide and is a major public health concern (1). It is thought that more than one billion people are intestinally colonized with ESBL-producing *Enterobacteriaceae* (ESBL-E) and that major drivers of the dissemination of these strains are antibiotic use in humans and animals and dissemination of antibiotic residues and ESBL-E in the environment (2, 3). Patients with infections caused by ESBL-E are known to suffer from increased morbidity and mortality compared to patients with infections caused by non-ESBL-E (4). *Escherichia coli* is the most common pathogenic bacterium among *Enterobacteriaceae* in

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human disease and causes serious and common infections such as septicemia and urinary tract infection (UTI) (5). *E. coli* is also the most commonly occurring ESBL-producing pathogen and has the gastrointestinal tract as its primary habitat (6). Secondary environmental habitats such as water and sediments are also known and the human oral cavity and oropharynx may also be considered important secondary habitats (6).

The large increase in the worldwide prevalence of CTX-M-type ESBL-E has been driven by horizontally transmitted genes carried by promiscuous plasmids and successful bacterial clones, but much is still unclear about the transmission of *Enterobacteriaceae* between hosts (7, 8). The presence of ESBL genes in the oral cavity, either harbored in *Enterobacteriaceae* or in other oral bacteria, might act as reservoirs of ESBL which may be shed to the intestinal tract and externally as airborne particles or through direct contact. Thus, oral carriage might influence important epidemiological parameters of ESBLs like duration of carriage and mode of transfer. While the fecal–oral route of transmission of these pathogens has been thoroughly explored in the literature, less attention has been given to airborne or oral–oral transmission.

Transient or longer term carriage of *Enterobacteriaceae* in the oral cavity or oropharynx has been linked to hospitalized patients and patients with periodontal disease, but may also be present in healthy individuals. One review of literature by Mobbs et al. found that the prevalence of oropharyngeal gram-negative bacilli in healthy individuals in 36 studies ranged from 0 to 61.1% (9). This study also sampled 120 individuals two times separated by 48 hours and found gram-negative bacilli in 36% of the cases, but in only 6.6% of the individuals the strains were identical between samples indicating a high frequency of transient carriage and thus rapid clearance of these species from the oral cavity and oropharynx. This may not to be the case in hospitalized patients and Johanson et al. found colonization rates up to 73% in severely ill (moribund) patients. In a more recent study Filius et al. also found that colonization was associated with severity of illness and in oropharyngeal samples it was 1.1% and 18% at admittance to general wards or intensive care units (ICU), respectively (10). The latter study also found that the prevalence increased significantly during and after hospitalization. Both the studies by Johanson et al. and Filius et al. suggest that oropharyngeal clearance of gram-negative bacilli is impeded in (severely) ill patients. Oral carriage of *Enterobacteriaceae* has also been associated with chronic periodontitis and an increase related to antibiotic use has been seen in this group (11–13). One would expect this increase to be even more pronounced for ESBL-E because of their (often multiple) resistance mechanisms. *Enterobacteriaceae* survive in air and on surfaces; thus, it is possible that the impact of airborne or oral–oral transmission of these bacteria has been underestimated – especially in high-risk situations in hospitals, but also in the general population (14).

Large studies have shown that the oral cavity includes several microbiological distinct niches with their own characteristic microbiota, including a wide variety of resistance determinants also encoding beta-lactamases (15–20). The oral microbiota is in constant interaction with the external environment and bacteria descending to the gut must pass through this area. Thus, a two-way exchange of resistance determinants between food and drink and the oral microbiota is likely to occur (18, 19). The oral cavity therefore possesses many characteristics necessary for a flexible transmission of genes, including ESBL-encoding genes from the environment to a host, between bacteria in a host or between hosts.

We aimed at determining the extent of oral carriage of ESBL in intestinal carriers of ESBL-E in a pilot study. We considered supragingival plaque the most likely oral location of ESBL-E and therefore sampled this area from known fecal carriers of *E. coli* carrying the ESBL gene $bla_{\text{CTX-M}}$ and examined these for ESBL-producing bacteria and $bla_{\text{CTX-M}}$. To our knowledge, the prevalence of oral carriage of ESBL in known intestinal carriers of ESBL-producing bacteria has not been investigated previously.

Materials and methods

Setting and design

As part of a study on fecal carriage of ESBL-producing *E. coli* or *Klebsiella pneumonia* associated with UTI, supragingival plaque and repeat fecal samples were collected from known fecal carriers of these bacteria. The study was conducted at the Department of Medical Microbiology at Vestre Viken Hospital Trust in Eastern Norway and the Department of Oral Biology, University of Oslo.

The study was approved by the South-Eastern Norway Regional Committee for Medical and Health Research Ethics following the Declaration of Helsinki principles (1975, 1983) (reference number: 2009/2037 BS-08901b). Written informed consent was obtained from all participants and donation of the supragingival plaque sample was not mandatory for participation in the other parts of the study.

Participants

The participants were adult volunteers from a study on fecal carriage of ESBL and risk factors for UTI caused by ESBL-producing *E. coli* or *K. pneumoniae* (21, 22). In this study, inclusion criteria were patients with community-acquired UTI caused by these bacteria or their household members. The patients were recruited after their urine had been submitted to a microbiological laboratory for analysis.

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Participants were interviewed; they submitted fecal samples and data were collected from the Norwegian Prescription Registry and patient medical records. During one time period in 2010 and one in 2011 participants with a fecal sample yielding ESBL-producing *E. coli* or *K. pneumoniae* were invited by mail to donate a supragingival plaque sample in a clinical setting. Periodontal status or antibiotic use was not known at the time of inclusion. The following exclusion criteria were used: 1) patients who had lived in Norway for <1 year, 2) were unable to answer our questionnaire, 3) had a previously diagnosed infection caused by ESBL-producing bacteria, and 4) patients with health care associated UTI (i.e. hospitalized or residing in a nursing home for >24 hours during the last 31 days). Sample collection was performed in two batches.

Sample collection and analysis

Fecal samples

Fecal samples were collected by the participants themselves using a sterile cotton swab applied to the toilet paper after defecation. The sample containers were returned by ordinary mail and cultured aerobically on ESBL selective agar plates (ChromID ESBL, BioMerieux, Marcy l'Etoile, France), enriched in a selective broth with 2.5 mg/ml of cefotaxime and then grown on the ESBL selective agar plates and on a non-selective lactose agar plate as growth control. Species identification was based on characteristic pink to burgundy oxidase-negative colonies for *E. coli.* Species identification of green colonies was obtained using the VITEK-2 system (GN ID card, BioMerieux). Genotypic verification of ESBL and phylogroup assignment of fecal ESBL-positive isolates was performed using polymerase chain reaction (PCR) as described earlier (23). Sub-typing of fecal ESBL-producing *E. coli* isolates was performed by ten-loci multiple-locus variable-number tandem-repeat analysis (MLVA) as described earlier (24, 25).

Oral plaque samples

Supragingival plaque samples were collected at the Department of Oral Biology, University of Oslo by a non-calibrated, but clinically experienced dentist with sterile Gracey-designed steel curettes (Stalan GMBH, Ahrensburg, Germany). Samples were taken from the mesio-buccal site of every tooth, with one vertical stroke in participants with their own teeth intact. Patients were not screened for periodontal disease site by site, but periodontal probing was done to make sure what pockets were healthy (<3 mm). Anamnestic occurrence of periodontal disease was recorded. From participants with dental prostheses or implants, biofilms were collected from these restorations. The samples were immediately transferred to one 5.5 ml vial of a pre-reduced anaerobically sterilized transport medium (Dental Transport Medium, Anaerobic System, Morgan Hill, CA) per patient (i.e. all samples from each patient were pooled). This is a medium in which also facultative organisms will survive. The samples were then homogenized within four hours by vortex and plated onto McConkey agar plates, ChromID ESBL (BioMerieux) and blood agar plates (nonselective trypticase soy agar supplemented with 5% defibrinated human blood, hemin 5 mg/ml, and menadione 0.05 mg/ml) and incubated at 37°C for 24–48 hours in aerobic (McConkey and ChromID ESBL) and anaerobic (90% N_2, 5% H_2, 5% CO_2) conditions (blood agar plates). Species identification of suspected *Enterobacteriaceae* was obtained using the VITEK-2 system (GN ID card, BioMerieux).

For PCR, 100 µl of the inoculated transport medium was diluted in 100 µl of Tris EDTA buffer and frozen at -70°C. DNA was extracted using MasterPure DNA Purification Kit (Epicentre, Madison, WI) according to the instructions of the manufacturer. PCR was performed in duplicate using the same procedure as for the fecal samples (23).

Sensitivity of PCR

To semi-quantitatively assess the sensitivity of the DNA extraction and PCR procedure described for oral plaque samples, 10-fold dilutions of CTX-M 1 producing *E. coli* (CCUG 55971) were inoculated in dental transport medium. DNA extraction and PCR were performed as described for the oral samples above. The detection limit of the DNA extraction- and PCR procedures from the dental samples were found to be 3–30 gene copies per PCR run indicating a lower detection limit of approximately 1,600–16,000 ESBL gene copies per supragingival plaque sampling procedure. The approximation assumed the same copy number of $bla_{\text{CTX-M}}$ genes in the clinical isolates as in the reference strain *E. coli* (CCUG 55971).

Results

Patient characteristics and results from culture- and molecular analyses of fecal and oral samples are presented in Table 1. A total of 17 participants were enrolled in the study. The mean age was 61 years (range 23–84) and 13 (76%) participants were female. The median Charlson index score was 1 and data from the prescription registry showed that seven out of 17 participants (59%) had been on one or more courses of antibiotics during the 3 months before the oral sample was obtained.

Fecal samples

Fecal carriage of ESBL-E was a prerequisite for inclusion and therefore present in all participants. At least one follow-up fecal sample was obtained after dental sampling from 14 of the 17 (82%) participants. The ESBL determinants in inclusion- and follow-up samples were harbored exclusively in *E. coli* and of the 17 inclusion isolates, 12 belonged to CTX-M phylogroup 1 and five to CTX-M phylogroup 9. The median duration from the inclusion sample to dental sampling was 64 days

Table 1. Background information about participants and time of fecal sample before and after the oral sampling procedure and results from these samples

ID	Age	Gender (F/M)	Number of days from collection of fecal sample to collection of oral sample	Number of days from oral sampling to next fecal sample	Phylogroup of CTX-M-genes from fecal sample before oral sampling[a]	Phylogroup of CTX-M-genes from fecal sample obtained after oral sampling[a]	MLVA results[b] (number of diverging loci before and after dental sampling)
1	64	M	8	81	1	1	0
2	65	F	11	81	1	1	0
3[c]	73	F	11	80	1	1	7
4	45	F	14	90	1	1	0
5	74	F	38	–	1	–	NA
6	52	F	44	13	9	9	0
7	23	F	56	16	1	1	4
8	84	F	57	13	1	Negative	NA
9[d]	69	F	64	6	1	1	0
10[e]	50	F	71	17	1	Negative	NA
11	68	M	74	726	9	Negative	NA
12	70	M	77	706	9	Negative	NA
13	59	M	100	–	1	–	NA
14	42	F	122	768	1	Negative	NA
15	75	F	148	29	1	1	0
16	45	F	186	3	9	Negative[f]	4
17	79	F	326	–	9	–	NA

ID: study participant number; F/M: female or male gender; CTX-M: Cefotaximase-Munich; MLVA: ten-loci multiple-locus variable-number tandem-repeat analysis; NA: not available.

[a]All the CTX-M genes identified prior to, and directly after oral sampling were harbored in *E. coli.* They were all assigned to phylogroups 1 or 9.

[b]MLVA was used for comparison of *E. coli* isolates in patients with more than one isolate. A low number of diverging loci indicate that the compared strains were identical or closely related.

[c]Patient had dental prosthesis.

[d]Patient had marginal periodontitis and four dental implants.

[e]Patient had marginal periodontitis.

[f]97 days later CTX-M 1 was detected and 145 days later CTX-M 1 and 9 were detected.

(range 8–326 days). The first follow-up sample from each participant was obtained at median 55 days (range 3–768 days) after dental sampling.

Results from 17 participants

1. In six (35%) participants, the same ESBL phylogroup and MLVA profile were identified in both the inclusion and follow-up sample.
2. In two (12%) participants with the same phylogroup, the MLVA profile diverged in four and seven loci.
3. In one (6%) participant, the same phylogroup was only detected in the third fecal sample after dental sampling and at that point the MLVA profile diverged in four loci.
4. Six (35%) participants did not donate a follow-up fecal sample or donated a sample more than 700 days after the dental procedure and all of these were negative.
5. Two (12%) participants donated fecal samples promptly after the dental procedure, but these and later samples were CTX-M-negative.

Of the nine fecal ESBL culture-positive isolates obtained after the dental procedure, seven isolates belonged to CTX-M phylogroup 1, one to CTX-M phylogroup 9 and in one participant both CTX-M 1 and 9 were identified.

Oral plaque samples

Of the 17 participants, 15 had their own teeth, one had a combination of own teeth and implants and one had a dental prosthesis only. Two patients had a history of marginal periodontitis. All oral samples were culture-positive on the blood agar plates. No ESBL-producing oral bacteria were detected by culturing on the ChromID ESBL.

Enterobacteriaceae were isolated from the McConkey cultures in three patients (17.6%). From these three participants, four different *Enterobacteriaceae* strains were

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identified, whereas none of the patients suffered from periodontitis. The strains were identified as *Rahnella aquatilis* (n = 1), *Pantoea* sp. (n = 2) and *Enterobacter cloacae* (n = 1).

Classical ESBLs were not identified by PCR in any of the oral plaque samples, but the *R. aquatilis* strain harbored the class A ESBL gene $bla_{\text{RAHN-1/2}}$ by PCR (26).

Discussion

We have investigated the oral cavity as a potential reservoir for the CTX-M-type ESBL resistance determinants in adults with fecal carriage of CTX-M producing *E. coli* using culture-based and molecular methods. The negative results obtained do not support the oral cavity as a significant reservoir of CTX-M-type ESBL.

The sensitivity of the molecular methods and ChromID ESBL agar plates used for detection of ESBL-E is high (27). Furthermore, we sampled supragingival plaque because this is the oral location we considered most likely to harbor *Enterobacteriaceae*. Thus, ESBL-producing strains or $bla_{\text{CTX-M}}$ should have been detected, if present in the oral samples from our participants. The negative results indicate the absence or presence in low concentrations only, of oral ESBLs in intestinal carriers and corroborate previous studies that have found a relatively low abundance of *Enterobacteriaceae* in the healthy oral cavity. The present study does not indicate that the oral carriage dynamics of ESBL-E is different from non-ESBL-E. The negative PCR results also suggest that classical CTX-M genes are not harbored in any of the many different oral bacterial species present in oral samples.

Our result on oral carriage elucidates the difference between fecal and oral carriage of ESBL-E. A recent study by Titelman et al. investigated the duration of fecal carriage after ESBL-E infections and found a gastrointestinal carriage rate of 66% three months after, and 43% one year after infection (28). The difference between oral and fecal carriage duration is probably a result of the oral cavity being a microbiological site distinctly different from the gastrointestinal tract. In one study, 509 different bacteria were detected in periodontal samples, but only 29 of these were found among the 560 intestinal- or fecal bacteria isolated, illustrating this point (29). Furthermore, to our knowledge only narrow spectrum beta-lactamases, AmpC beta-lactamases or unspecified TEM-genes have been detected in plaque of patients with periodontitis or in plaque samples from the normal population (20, 30–32). The *R. aquatilis* with the ESBL gene $bla_{\text{RAHN-1/2}}$ identified in the present material is thus, to our knowledge, a novelty. However, *R. aquatilis* is probably a transient bacterium in the mouth originating from drinking water (33). Supporting this, it was not identified in the patient's fecal samples, and this finding does not change the overall picture of intestinal ESBL carriage as not related to oral carriage.

The present study does not support the oral cavity as a source of transmission of ESBL. But worryingly, after doxycycline therapy more than a 10-fold increase was seen in subgingival numbers of *Enterobacter aerogenes* and *E. coli* (13). Another study showed an increasing frequency of nasal and pharyngeal carriage of *Enterobacteriaceae* during the hospitalized period in patients admitted to general wards and intensive care units (10). Thus, ESBL-E may under certain circumstances be more frequent in the oropharyngeal area than our study demonstrated. Possible transmission related to oropharyngeal carriage was demonstrated in another study by Branger et al., which identified an outbreak of ESBL-E possibly related to a poorly maintained bronchoscope (34). Thus, there are suggestions that periods of oral- or upper airway carriage may facilitate the transfer of ESBL-E, but this remains to be documented. In hospitals, where oral and oropharyngeal carriage appears to be more frequent, this may have serious consequences due to inter-patient transmission. The present results apply to non-hospitalized fecal carriers of ESBL-E and should not discourage infectious control measures that take into account a possible oropharyngeal source of ESBL-E (10).

Our study has limitations of which the most important is its small size. Furthermore, the intestinal colonization status of the participants at the exact time of the dental sampling procedure was unknown since fecal samples were not collected at that time. However, ESBL was detected in 9 of 11 (82%) participants that donated a fecal sample within 90 days after dental sampling. This is in line with published data on the duration of fecal ESBL carriage after infection (28). The exact same ESBL or *E. coli* MLVA sub-type was not identified in all nine participants. This may be explained by re-colonization and thus it is a possibility that these participants were not fecal carriers at the time of the dental procedure. Since Norway is a country with a low prevalence of ESBL, this seems unlikely. Other explanations like initial co-colonization with several ESBLs, transfer of plasmids between *E. coli* strains and ESBL below the detection threshold seem better explanations for diverging phylogroups and sub-types before and after dental sampling.

It's not possible to know how many participants were actually fecal carriers at the time of oral sampling, yet an estimate based on the 11 participants who did donate a fecal sample within 90 days of the oral sampling is 14 of 17. Given that 20% of fecal ESBL-E carriers are also oral carriers, the chance of a negative result in 14 individuals is less than 5% ($0.80^{14} < 0.05$), indicating that the true oral carriage rate in fecal ESBL-E carriers is lower than this.

Furthermore, none of the 17 previously known fecal carriers of ESBL were found to be oral carriers at a median of 64 days after fecal sampling. At this point in time, at least 66% of patients infected with ESBL-E would be expected to be fecal carriers according to one earlier

study (28). Thus, we may make a cautious conclusion that the frequency of fecal carriage is significantly higher than oral carriage after a UTI caused by ESBL-E ($p < 0.001$, Fisher's exact test). The results do not rule out transient oral ESBL-carriage in fecal carriers or oral carriage in high-risk groups like hospitalized patients. Because of the limitations of this pilot study and the importance of the issue investigated, further studies of oral carriage of ESBL are warranted.

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Conflict of interest and funding

The authors have no conflict of interest.

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